Pro-oxidant effects of cross-linked haemoglobins explored using liposome and cytochrome *c* oxidase vesicle model membranes

Melanie S. ROGERS,* Rakesh P. PATEL,† Brandon J. REEDER,† Paolo SARTI,‡ Michael T. WILSON† and Abdu I. ALAYASH*§

*Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20815, U.S.A., †Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester, Essex, CO4 3SQ, U.K., and ‡Institute of Biological Chemistry, University of Cagliari and CNR Centre of Molecular Biology, Rome, Italy

The therapeutic use of cell-free haemoglobin as a blood substitute has been hampered by toxicological effects. A model asolectin (phosphatidylcholine/phosphatidylethanolamine) liposome system was utilized to study the pro-oxidant efficiency of several chemically modified haemoglobins on biological membranes. Lipid peroxidation, resulting from the interactions between haemoglobin and liposomes, was measured by conjugated diene formation and the maximal rates of oxygen uptake. Spectral changes gave insight into the occurrence of the ferryl iron species. The residual reactivity of oxidatively damaged haemoglobins with ligands during incubation with liposomes was assessed from rapid kinetic carbon monoxide-binding experiments. Liposomes in which cytochrome *c* oxidase was embedded show both haemoglobin and the enzyme to be oxidatively damaged during incubation. The functional state of cytochrome *c* oxidase was monitored in the presence and absence of a free radical scavenger. Once in contact, both unmodified and modified haemoglobins triggered and maintained severe radical-mediated membrane damage. Differences in the pro-oxidant activities among haemoglobins may be explained by either the differential population of their ferryl intermediates or disparate dimerization and transfer of haem into the membrane with subsequent haem degradation. This study may contribute to a better understanding of the molecular determinants of haemoglobin interactions with a variety of biological membranes.

INTRODUCTION

Haemoglobin is able to transport oxygen effectively within the optimum environment provided by the erythrocyte. Several systems (e.g. ferric haemoglobin reductase, catalase, superoxide dismutase, glutathione) are present to retain haemoglobin in its functional ferrous form and to prevent damage induced by partially reduced oxygen derivatives. Even with these protective mechanisms, around 1–2% of the total cellular haemoglobin is oxidized at any given time [1]. Containment by the erythrocyte membrane also maintains the high organic phosphate concentration essential for haemoglobin function. Furthermore, encapsulation within the erythrocyte prevents potential damage to the kidney caused by tetramer dissociation to dimers [2].

Acellular haemoglobin, in the absence of 2,3-diphosphoglycerate has a high oxygen affinity and, at micromolar concentrations, a predisposition to rapid dimerization. Several chemical and genetic strategies have successfully overcome these limitations in the use of haemoglobin solutions as oxygen-carrying red-cell substitutes (for review see [3]). Chemical modifications to form intramolecular and/or intermolecular cross-links are widely used and have resulted in stable haemoglobin-based oxygen carriers with oxygen affinities approaching that of whole blood and prolonged intravascular retention [3]. One of the widely used intramolecular cross-linking agents is bis(3,5-dibromosalicyl)fumarate (DBBF). When oxyhaemoglobin is reacted with this reagent it cross-links the β chains between the Lys^{82} residues producing a derivative (β-DBBF) which is stable to dissociation but has an oxygen affinity close to that of unmodified HPLC-prepared human haemoglobin A (HbA_o) [4]. Conversely when deoxyhaemoglobin is employed a stable low-oxygen-affinity derivative is produced in which the two Lys^{99} residues of the α subunit are linked (α-DBBF). Due to these favourable characteristics, α-DBBF has been developed as a blood substitute [5]. Polymerization of α-DBBF by bis(maleolglycylamide)poly(ethylene glycol) (BMMA-PEG), yielding poly-α-DBBF, offers the added benefit of reduced renal clearance and increased retention in the vascular circulation [6].

Recent work on these products has shown that in addition to modifying ligand interaction properties, site-specific modification can also affect the tendency of some of these haemoglobins to undergo oxidative modifications when challenged by hydrogen peroxide (e.g. the production of the ferryl species) [7–9]. Due to the potential toxicity of cell-free haemoglobin, attention is now focused towards the design of proteins which are stable towards autoxidation, oxidative damage and lipid peroxidative activity [10].

The extent to which acellular haemoglobin leads to peroxidation of biological membranes *in vivo* is unknown. In this study the pro-oxidant effect of a number of modified haemoglobins on biological membranes is investigated by using liposomes as model membranes. These structures are well-characterized and are amenable to the measurement of the indices of lipid peroxidation and thus can give insight into the nature of the pro-oxidant action of chemically modified haemoglobins developed as blood substitutes. Liposomes are easier to use than erythrocyte membranes as the constituents of the model membrane can be defined and easily manipulated. Liposomes, although artificial, may have proteins incorporated into their bilayers, e.g. cytochrome *c* oxidase [11]. This enables further investigation of the effects of haemoglobin-induced active oxygen radicals and/or lipid peroxidation on the activities of membrane-

Abbreviations used: α-DBBF/β-DBBF, deoxy/oxy HbA cross-linked with bis(3,5-dibromosalicyl)fumarate (DBBF); BMAA-PEG, bis(maleoylglycylamide)poly(ethylene glycol); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; COVs, cytochrome c oxidase vesicles; DBBF, bis(3,5-dibromosalicyl)fumarate; HbA, haemoglobin A; HbA_o, HPLC-prepared human HbA; poly-α-DBBF, α-DBBF polymerized with bis(maleoylglycylamide)polyethylene glycol; RCR, respiratory control ratio.

§To whom correspondence should be addressed.

bound enzymes and to understand the molecular mechanisms through which oxidative stress induces changes in biological membranes. In this study, we have used phospholipids purified from soybean asolectin containing predominantly equimolar amounts of phosphatidylcholine and phosphatidylethanolamine (and trace polyunsaturated fatty acids). The liposomes produced are the most convenient to properly incorporate cytochrome *c* oxidase into vesicles displaying the same functional properties if the enzyme were *in situ* [12,13].

The study of haemoglobin oxidation in the presence of phospholipids is also important in the development of haemoglobin-containing liposomes as an oxygen-carrying medium.

Consistent with previous studies [14], we show that reactions between haemoglobin and liposomes or cytochrome *c* oxidase vesicles (COVs) affects the properties of both the haemoglobin and the lipid membrane. Under these conditions chemically modified haemoglobins appear to be less effective in inducing phospholipid peroxidation of membranes and in altering their permeability.

EXPERIMENTAL

Materials

All biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. As a precaution against free Fe ions, all solutions were prepared in deionized water (18 mΩ). EDTA was also included in the solutions to chelate any adventitious Fe ions.

HbA_0, α-DBBF and β-DBBF were gifts from the Letterman Army Institute of Research, San Francisco (presently located at Walter Reed Army Institute of Research, Washington, D.C., U.S.A.). HbA_0 was prepared as previously described [15]. α-DBBF and β-DBBF were produced according to [5]. Poly-α-DBBF, prepared according to [6], was a kind gift from Baxter Corp., IL, U.S.A. All haemoglobin solutions were chromatographed on a DEAE-Sephadex column to ensure removal of catalase, superoxide dismutase and glutathione. The high level of purity of the haemoglobin solutions was confirmed by the use of SDS/PAGE analysis, isoelectric focusing and HPLC.

Oxidized (met) forms of haemoglobins were obtained by the addition of a 1.2:1 molar ratio of ferricyanide:haem. Excess ferri- and ferrocyanide were removed by a two-step Sephadex G-25 gel filtration using firstly 50 mM Hepes buffer/1 M NaCl, at pH 8.3, followed by 50 mM Tris buffer at pH 8.3. Cyanomethaemoglobins were produced according to [16]. Haemoglobin concentrations were calculated using appropriate absorption coefficients as reported in [1].

Liposome preparation

Phospholipids (50 mg/ml) purified from soybean by acetone–ether fractionation [17] were stirred in 100 mM Hepes, pH 7.4, for 1 h under nitrogen gas at 4 °C. Liposomes were prepared by either sonication (40% duty cycle, microtip limit 3–4) or extrusion through a 100 nm polycarbonate membrane mounted in a small volume extrusion apparatus [18].

Cytochrome *c* oxidase vesicle (COV) preparation

Bovine heart cytochrome *c* oxidase was purified by the method of Yonetani [19]. Enzyme purity was monitored spectrophotometrically. It was considered pure when, in a reduced form, the amplitude of the absorption peaks at 560 nm and 550 nm, corresponding to cytochrome *b* and cytochrome c_1 respectively, were less than 5% of that of the oxidase peak at 605 nm.

Cytochrome *c* oxidase was incorporated into liposomes to form COVs according to the cholate dialysis method of Hinkle et al. [11]. Bovine heart cytochrome *c* oxidase (10 μM total haem) was added to a lipid solution which had been sonicated to clarity and dialysed extensively against buffer solution to remove detergent. Enzyme activity was measured polarographically in the presence of sodium ascorbate (10 mM), cytochrome *c* (20 μM) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (200 μM). The respiratory control ratio (RCR) was calculated as the ratio of enzyme activity in the presence and absence of valinomycin and nigericin [or carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP)] [20].

Haemoglobin–liposome interactions

Oxy-, met- and cyano-met forms of haemoglobin (10 μM) were incubated with liposomes (0.6 mg/ml) prepared by sonication in 100 mM Hepes/0.1 mM EDTA, pH 7.4, at 37 °C according to [14]. Spectral changes were monitored in the Soret and visible regions of the absorption spectrum. The optical cuvette was equipped with a tightly fitting oxygen electrode to allow spectral and polarographic data to be collected simultaneously on the same sample.

Occurrence of the ferryl species

The occurrence of the ferryl species was explored in both native methaemoglobin and the chemically modified methaemoglobins (75 μM) during incubation with liposomes (4.6 mg/ml) prepared by extrusion in 100 mM Hepes buffer/0.1 mM EDTA, pH 7.4, at 37 °C. Samples of the reaction mixture (10 μM final haem concentration) were taken at known time points, mixed with sodium sulphide (2 mM) and observed spectrally over the range 380 to 700 nm. Spectrophotometric determinations were carried out either using a Cary 5E or Hewlett Packard 8452A diode-array spectrophotometer.

Residual ligand binding ability during incubation with liposomes

Methaemoglobins were incubated with liposomes (0.6 mg/ml) prepared by sonication in 100 mM Hepes buffer/0.1 mM EDTA, pH 7.4, at 37 °C. Aliquots were taken at specific time intervals and a few grains of sodium dithionite were added in order to reduce the haemoglobin (6.7 μM to 10.5 μM before mixing). The carbon monoxide-binding ability of the reduced haemoglobin was measured under pre-steady-state conditions at 25 °C by rapidly mixing the sample with carbon monoxide dissolved (200 μM before mixing) in 100 mM Hepes/0.1 mM EDTA, pH 7.4, using an Applied Photophysics (U.K.) stopped-flow spectrophotometer.

Haemoglobin–COV interactions

Haemoglobin effects on biological membranes were simulated by incubating oxy- or met-haemoglobin with COVs [14]. In some experiments, α-tocopherol (100 μM) was added to the lipid solution prior to production of the liposomes. Consequences of the addition of this antioxidant were assessed from RCR measurements of the COVs in the sample [14].

RESULTS

A previous study by Sarti et al. [14] has shown that the ferric rather than the ferrous form of haem iron was the more potent activator of haemoglobin-induced oxidative changes in liposomes, thus experiments were conducted using methaemoglobin rather than oxyhaemoglobin. Furthermore, the susceptibility of chemically modified haemoglobins to autoxidize may result in an

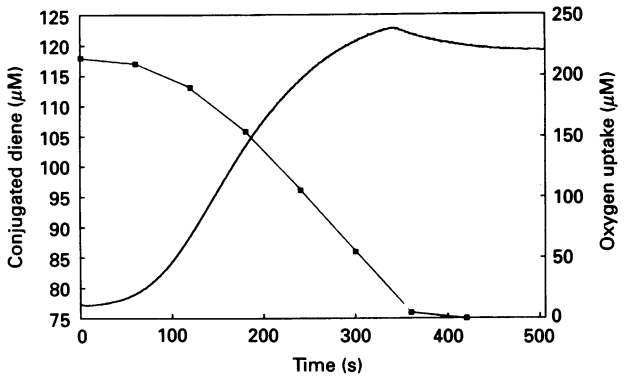

Figure 1 Time courses of oxygen uptake and conjugated diene formation occurring during the incubation of metHbA$_0$ with liposomes

MetHbA$_0$ (10 μM) was incubated with liposomes (0.62 mg/ml) in 0.1 M Hepes/0.1 mM EDTA, pH 7.4, at 37 °C. ■ represents points taken from the polarographic trace and the line represents conjugated diene formation calculated using ϵ_{234} 24400 $M^{-1} \cdot cm^{-1}$.

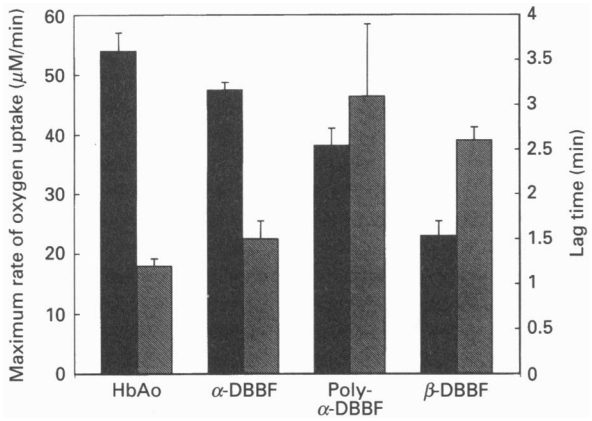

Figure 2 Lipid peroxidation in liposomes incubated with methaemoglobins

Methaemoglobins (10 μM) were incubated with liposomes (0.62 mg/ml) in 0.1 M Hepes/0.1 mM EDTA, pH 7.4, at 37 °C. The open and hatched areas refer respectively to the maximum rate of oxygen uptake measured polarographically and the lag times calculated from conjugated diene formation measured by the increased absorbance at 234 nm.

accumulation of met haem *in vivo* [7,8,21]. On adding the ferric forms of HbA$_0$ and its cross-linked derivatives to suspensions of liposomes both the haemoprotein and the lipid constituents of the system underwent chemical change. Figure 1 shows typical profiles of oxygen uptake measured polarographically and of conjugated diene formation (monitored at 234 nm), both indicative of lipid peroxidation [14]. These time courses are essentially identical and are characterized by a 'lag' and 'propagation' phase typical of radical-mediated reactions [14]. The length of the lag phase was determined from the intercept of the tangent taken to the lowest rate of oxidation with the tangent of the rate during propagation, i.e. highest rate of oxidation [14]. Once all oxygen dissolved in the buffer had been consumed conjugated diene formation ceased, as is indicated by the lack of further increase in absorbance at 234 nm. The maximum rates of oxygen uptake determined from the maximum slope (Figure 1) and the lag time for conjugated diene formation for the different methaemoglobin derivatives are shown in Figure 2. The haemoglobins are listed in order of decreasing maximal oxygen uptake rates HbA$_0$ > α-DBBF > poly-α-DBBF > β-DBBF. With the exception of poly-α-DBBF this is the same as the order of increasing lag time, i.e. of decreasing activity as a pro-oxidant. The three chemically modified haemoglobins have longer lag times and lower rates of oxygen uptake than metHbA$_0$ and are thus poorer pro-oxidants in this system.

Incubation of the oxyhaemoglobins (10 μM) with the liposomes also resulted in oxygen consumption and conjugated diene formation. The time courses were slower than those observed with the respective methaemoglobin derivatives (results not shown). The complex reaction between oxyhaemoglobin and liposomes appears to be mediated by the small but unavoidable methaemoglobin contamination (5–8%) [14]. Experiments performed with cyano-met derivatives of these haemoglobins show greatly reduced pro-oxidant activity (results not shown).

From Figures 1 and 2 it is seen that addition of haemoglobin and its derivatives leads to extensive lipid peroxidation. The reciprocal effects on the haemoglobins are shown in Figure 3 where the absorbance spectra of the haemoproteins are shown during the course of their reaction with liposomes. The most prominent feature is a decrease in intensity. There is also evidence for the formation of at least one additional discrete spectral species, which is most obvious in met-α-DBBF where a shoulder centred around 420 nm is seen to develop (Figure 3b). A plot of absorbance at 423 nm, the wavelength of maximum absorbance change in this region, versus time reveals the varying extent to which this species is populated in these haemoglobins. (Figure 4a). The control HbA$_0$, after a lag phase, shows an overall decrease in absorbance, while β-DBBF and poly-α-DBBF exhibit an intermediate time course possibly due to comparable rates of formation and decay of this spectral species. A possible identity for the species seen with α-DBBF is haemoglobin containing ferryl iron [22]. This is further supported by the appearance of an absorbance peak at 620 nm on combination with sodium sulphide (Figure 4b), a classic test for the presence of ferryl haemoglobin species [23]. Under identical conditions, no such peak was observed with the haemoglobins other than α-DBBF. The reaction of ferryl [Fe(IV = O)] iron with sodium sulphide (Na_2S) forms sulphhaemoglobin. This sulph-haem complex has an absorbance maxima at 620 nm. This contrasts with the reaction of ferric haem with sulphide which produces a compound with a different distinctive absorbance maxima [24]. The reaction of sulphide with ferryl iron results in the reduction of a β-carbon double bond in one of the pyrroles in protoporphyrin IX to form a chlorin [25]. The higher oxidation state of the ferryl haem iron withdraws electrons from the β–β bond, rendering it more susceptible to nucleophilic attack by HS^-. Singular value decomposition (SVD) and non-linear least-squares kinetic analysis [8,26] of the reaction of met α-DBBF with liposomes (Figure 3b) revealed one of the reconstructed spectral components to have an absorbance maxima in the Soret region of 420 nm (results not shown) which is typical of a ferryl haem species.

The decrease in intensity in all the spectra shown in Figure 3 results from deconjugation of the porphyrin ring with progressive haem degradation and possible loss from globin [27,28]. The spectrum of haemin chloride did not change during incubation with liposomes. The iron group of haemin chloride is blocked by chloride and may thus be sterically unable to participate in oxidative reactions. Ferrocytochrome *c* experienced a slight reduction (11%) as monitored at 550 nm (results not shown). Ferricytochrome *c* manifested no change in its spectrum. The haem group of cytochrome *c* is covalently linked and the slight reduction may be by virtue of its exposed haem edge contacting lipid and being reduced in an electron transfer reaction [29]. This

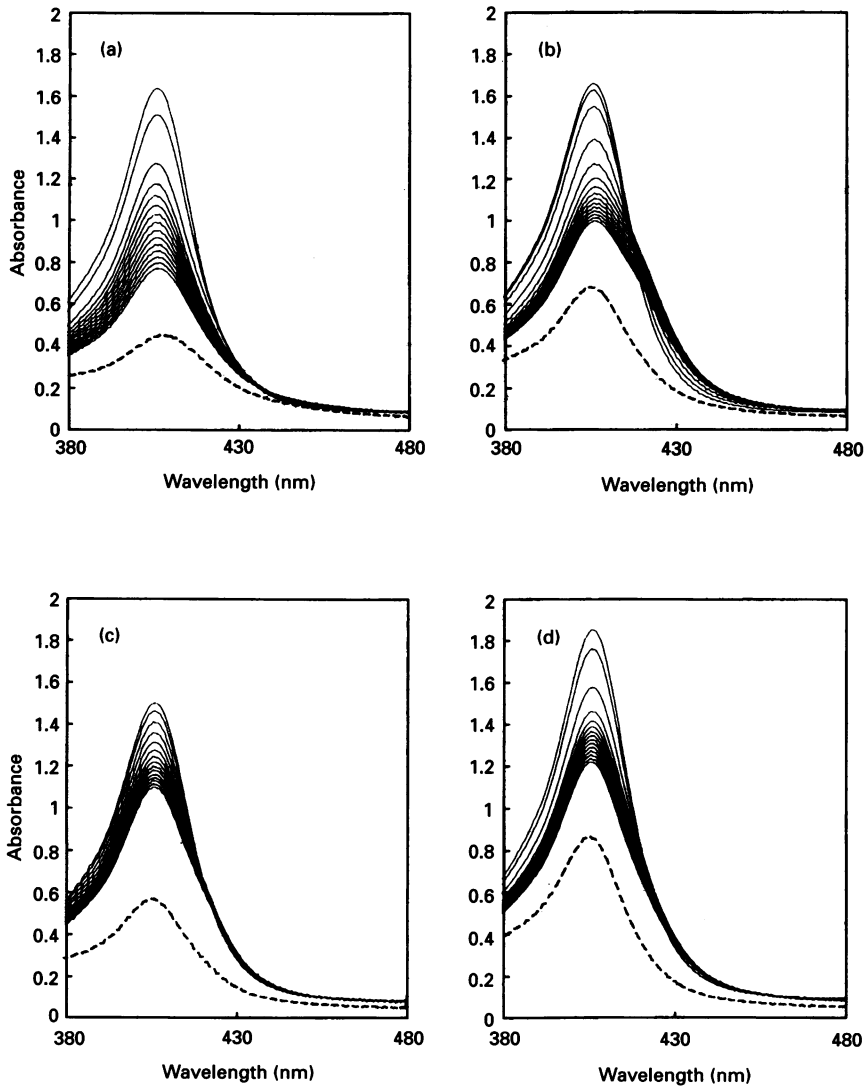

Figure 3 Absorbance changes in the Soret region during incubation of methaemoglobins (10 μM) with liposomes

Methaemoglobins (10 μM) were incubated with liposomes (0.62 mg/ml) prepared by sonication in 0.1 M Hepes/0.1 mM EDTA, pH 7.4, at 37 °C. Spectra were recorded every 2 min for 28 min. A dashed line represents spectra recorded at later time points as indicated. (**a**) HbA_0 (- - -, 84 min); (**b**) α-DBBF (- - -, 150 min); (**c**) β-DBBF (- - -, 158 min); (**d**) poly-α-DBBF (- - -, 138 min).

further supports the hypothesis that either unblocked iron or free haem may play a role in the mechanisms in action during liposome–haemoglobin incubations.

The functional state of the haemoglobin was monitored by rapid kinetic measurement of the carbon monoxide combination rate in the presence of dithionite. Aliquots of the reaction mixture containing liposomes and methaemoglobin were taken at known time intervals and the carbon monoxide combination time courses determined by stopped-flow spectrophotometry. Lipid does not have an absorbance contribution at the monitoring wavelengths so only haemoglobin changes were recorded (see Figure 5). Both the shape and amplitude of the time courses are seen to change as a consequence of incubation. The initial autocatalytic shape characteristic of native HbA_0 becomes exponential and faster on prolonged exposure to liposomes. The second-order rate constant for carbon monoxide combination increases from approx. 1.5×10^5 $M^{-1} \cdot s^{-1}$ at the beginning of incubation to approx. 10^6 $M^{-1} \cdot s^{-1}$ after 45 min for the fast phase of carbon monoxide combination. This upper value is approaching that for the free chains [1]. These changes suggest a loss of normal haemoglobin due to weakened haem–haem interactions and to haem destruction.

In Figure 6 we compare the loss of absorbance of the Soret band (Figure 3) with the decreasing amplitude of the signal for carbon monoxide combination (Figure 5). A number of features of this comparison may be noted. First, the two processes clearly reflect a common underlying mechanism, the time courses for each individual haemoglobin derivative being the same in the different experiments with respect to the order of effectiveness; i.e. HbA_0 > α-DBBF = poly-α-DBBF > β-DBBF, similar to that exhibited in Figure 2. Secondly, the time course in the decay of HbA_0 is complex, comprising a lag phase followed by a biphasic decay. Comparison with the cross-linked haemoglobins reveals that the faster process of the decay is less evident and the haem destruction following the lag phase is largely through the slower process seen with HbA_0.

As a parametric index of membrane damage, changes in the permeability properties of the liposomal membrane induced by

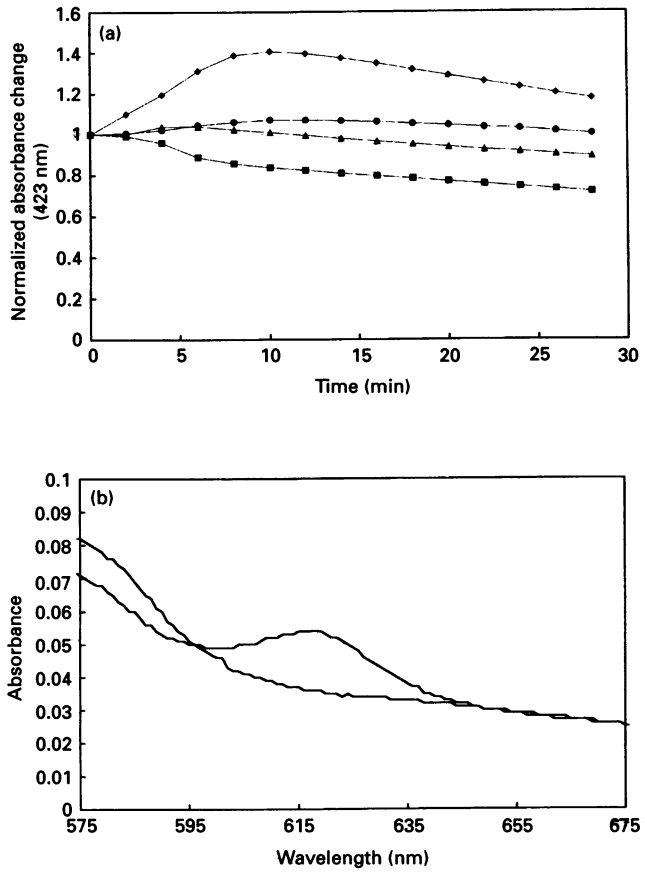

Figure 4 (a) Absorbance changes at 423 nm occurring during incubation of methaemoglobins with liposomes and (b) the occurrence of the ferryl species in α-DBBF

(**a**) Experimental conditions as in Figure 3. ■, HbA_0; ◆, α-DBBF; ●, β-DBBF; ▲, poly-α-DBBF. (**b**) Experimental conditions as in Figure 3, except liposomes prepared by extrusion were employed. Aliquots of the reaction mixture (10 μM final haemoglobin concentration) were taken every 20 min and combined with sodium sulphide (2 mM). The spectra shown were recorded at $t = 0$ min (lower) and $t = 20$ min (upper).

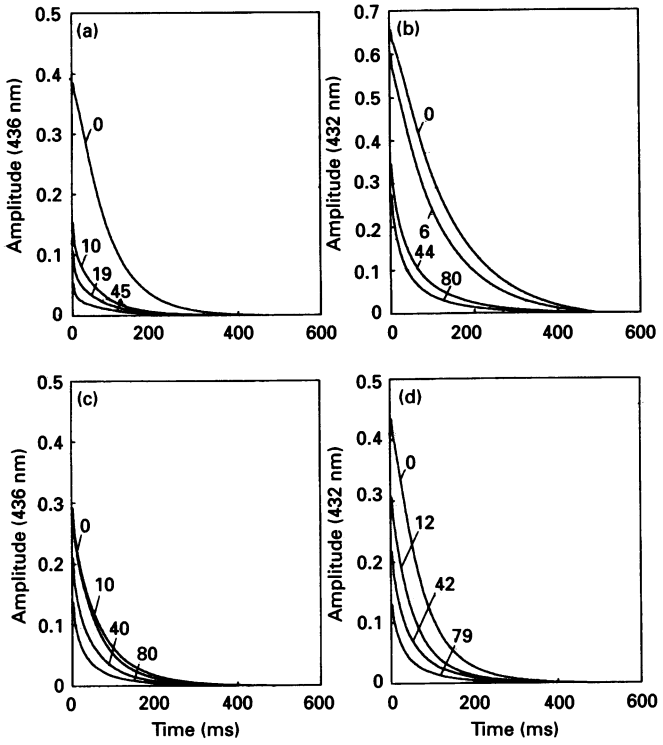

Figure 5 Time courses of carbon monoxide binding to ferrous haemoglobins recorded at various times of incubation of the met form with liposomes at 37 °C

A solution of CO gas (200 μM before mixing) was rapidly mixed with aliquots of haemoglobin:liposome mixture (6.7 μM to 10.5 μM haemoglobin before mixing) in the presence of dithionite in 0.1 M Hepes/0.1 mM EDTA, pH 7.4, at 25 °C. (**a**) HbA_0, (**b**) α-DBBF, (**c**) β-DBBF, (**d**) poly-α-DBBF.

incubation with haemoglobin were studied using liposomes in which cytochrome *c* oxidase had been incorporated into the membrane (COVs) [11]. In such a system the activity of the enzyme is a function of the permeability of the membrane to cations [20]. The ratio of the activity of the enzyme in the presence to that in the absence of added ionophores (i.e. valinomycin plus CCCP) is termed the RCR, the higher the value of this ratio the more impermeable is the membrane to ions. Figure 7 shows the dependence of the RCR on incubation time with the haemoglobin derivatives. In agreement with earlier reports [14] methaemoglobin is seen to render the liposome membrane more permeable to cations as the incubation is lengthened.

In accord with the results in Figures 2 and 3 which showed that the cross-linked haemoglobins were less effective than HbA_0 as pro-oxidants, we see this behaviour is repeated in the experiments reported in Figure 7. With the exception of poly-α-DBBF at short incubation times, native HbA_0 is the most effective derivative in decreasing the RCR.

Addition of vitamin E to the lipid prior to COV formation inhibits lipid peroxidation leaving the RCR largely unchanged (87% of control). As vitamin E is known to act as a peroxyl radical scavenger we may conclude that the loss of RCR is a consequence of lipid peroxidation that results in elevated cation permeability [30].

DISCUSSION

Several acellular haemoglobin preparations are now under intense evaluation as potential oxygen-carrying red-cell substitutes intended for a variety of clinical applications [3]. The unexpected toxicity exhibited *in vitro* and *in vivo* by these modified haemoglobins, however, represents a major impediment to progress in development of a viable blood substitute. Iron-mediated oxygen radical formation has been implicated in the cellular injury and cytotoxicity observed when these proteins are used as reperfusion agents [31,32]. The proximity of circulating stroma-free haemoglobin to vascular endothelium led many in the field to suggest that haem intercalation into membranes and/or degradation of membrane phospholipids following peroxidation have possible roles in the oxidative stress of endothelium [33,34]. In this regard the oxidation state of the iron in the haemoglobin molecule may play a crucial role in induction of endothelial cell injury. Balla et al. [33] found that methaemoglobin is a more potent sensitizer of endothelial cells to hydrogen peroxide than ferric forms of myoglobin or cytochrome *c*. The ferryl form of a number of modified bovine haemoglobins have recently been shown to promote endothelial membrane oxidation and peroxidation [35].

In agreement with earlier reports [36,37] we find that methae-

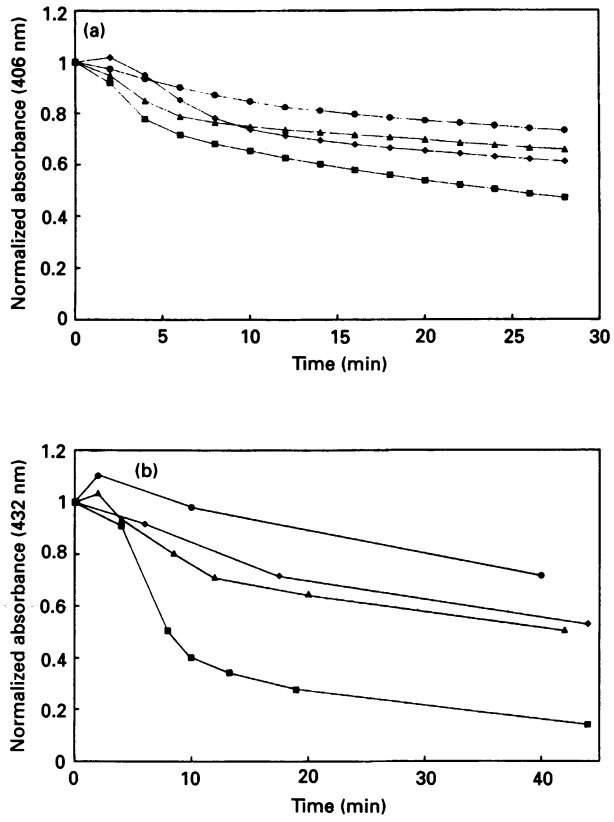

Figure 6 The comparison between spectral changes in the Soret region (406 nm) taken (a) from Figure 3 and (b) amplitude changes upon carbon monoxide combination

For experimental conditions see Figures 3 and 5.

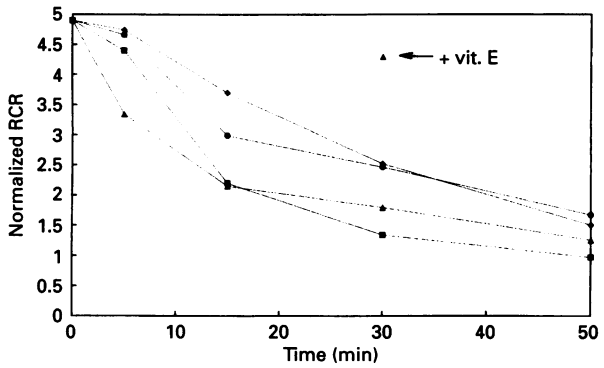

Figure 7 Respiratory control ratio (RCR) of cytochrome *c* oxidase vesicles measured during incubation with methaemoglobins

■, HbA_0; ◆, α-DBBF; ●, β-DBBF; ▲, poly-α-DBBF (▲ ← + vit. E, measured with the addition of 100 μM α-tocopherol). Experimental conditions are described in Figure 1.

moglobins are highly effective pro-oxidants for the phospholipid membrane causing rapid peroxidation and changes in functional properties. Sarti et al. [14] and Paganga et al. [38] have concluded that the initial reaction is between endogenous hydroperoxides present in the membrane and haemoglobin in a ferric (met) oxidation state. The resultant ferryl species can itself further react with peroxides to regenerate ferric iron and yield peroxyl radicals. This ferric-ferryl cycle results in lipid-phase radicals (LOO·, LO·) which propagate the peroxidation reaction throughout the membrane. Our results are in general agreement with this conceptual framework, cyanide inhibiting redox cycling of ferric iron and vitamin E acting as a peroxyl radical scavenger [14]. Our findings that liposomes produced by an extrusion process under nitrogen rather than by sonication are much more resistant to the peroxidative action of haemoglobins is fully consistent with the notion that endogenous hydroperoxides were an essential requirement for the reaction to start. Endogenous hydroperoxide levels are variable in lipid preparations and are also increased during the sonication process in comparison with extrusion methods of liposome production. Additionally, production of the ferryl species depends on the amount of endogenous hydroperoxides present in the liposomes. This explains the longer time of production of detectable ferryl species seen in Figure 4(b) for extruded liposomes compared with that seen in Figure 4(a) using sonicated liposomes.

The central purpose of this investigation was to compare the pro-oxidant activities of HbA_0 with a variety of cross-linked haemoglobins which may find use as therapeutic agents. In all the experiments reported here the modified haemoglobins were significantly less able to instigate the peroxidation of lipids than HbA_0. Similarly the haem group of these modified proteins was more resistant to breakdown than in HbA_0. This finding is interestingly different to that reported for the interaction between these proteins and hydrogen peroxide where the α-DBBF derivative consistently showed greater sensitivity to breakdown than HbA_0 [7,21]. It has recently been suggested that the cycling between the ferric and ferryl haem of a number of modified haemoglobins in the presence of hydrogen peroxide can result in an apparent peroxidase activity and that this activity is sensitive to structural modifications of the protein [8,39]. Haemoglobin is also able to catalyse the oxidation of unsaturated fatty acids, such as phosphatidylcholine [40]. Haem breakdown in the proteins under study here have similar kinetic profiles as indexed by spectral decay and loss of ligand-binding ability, although they are proportionally different. Lipid peroxidation occurring in parallel with haemoglobin oxidation suggests that a close link exists between these processes.

Although the modified haemoglobins may, as a group, be distinguished from HbA_0 it is more difficult to rationalize fully the relative effects of the protein in the different experiments we report. The system is sufficiently complex to resist facile analysis but some salient features may be worthy of mention.

(a) The ferryl form of the haemoglobin, postulated as an important intermediate, seemed in general to be populated at levels hard to detect under the optical conditions prevalent in liposomal suspensions. This is likely to result from differing rates of formation and decomposition of this species [8]. However, in the α cross-linked haemoglobin we have reasonable evidence for the existence of significant amounts of this species during the reaction (Figure 3b).

(b) The decay of the haem spectra and carbon monoxide-binding capacity (Figures 6a and 6b) contain rapid phases with HbA_0 which are less obvious with the modified cross-linked haemoglobins. One explanation is that these processes (and hence by implication the peroxidative reaction also) are mediated via haemoglobin dimers. HbA_0 at these concentrations will exist in a dimer/tetramer equilibrium (K_d approx. 3 μM) whereas the cross-linked derivatives will not [41,42]. It is possible that dimers are more effective than tetramers in the reaction we describe, which would account for the distinction between HbA_0 and the cross-linked proteins [42,43].

Additionally, it has been reported that interactions of haemoglobin or haemin with phosphatidylcholine and phosphatidylserine result in haem transfer from the protein to the lipid phase [37,44]. We examined this by observing the spectrum of liposomes separated from free haemoglobin by centrifugation. Haem was not detected in the lipid phase. This does not rule out, however, the occurrence of haem transfer. Undetectable levels of haem (1–2 %) may still be able to cause lipid peroxidation by the catalytic decomposition of lipid hydroperoxides. Thus the relative ease of haem loss by native and chemically modified haemoglobin may be an important factor in their differing pro-oxidant effects. In support of this, haem transfer experiments testing the stability of haem–globin interactions of unmodified and two chemically modified haemoglobins by measuring the haem exchange between the ferric forms of the protein and albumin have been performed [41,45]. Preliminary data (to be published elsewhere) revealed biphasic rates [k_f (fast phase); k_s (slow phase)] of haem loss from β- and α-chains; for HbA_o ($k_f = 0.34 \pm 0.09$ min^{-1}, $k_s = 0.0140 \pm 0.0005$ min^{-1}), α-DBBF ($k_f = 0.20 \pm 0.05$ min^{-1}, $k_s = 0.0100 \pm 0.0004$ min^{-1}) and poly-α-DBBF ($k_f = 0.120 \pm 0.033$ min^{-1}, $k_s = 0.014 \pm 0.003$ min^{-1}). Although not carried out under physiological conditions (pH 9.0, 20 °C), these experiments provide some index of the ease with which haemoglobins lose haem.

Many of the reported toxicological effects of haemoglobin solutions *in vivo* may be explained by the interaction of the protein with the vascular endothelium, perhaps by direct intercalation of haem into the endothelium [33]. Concurrently, haemoglobin can liberate unsaturated fatty acids from the endothelial cell membrane which could result in increased oxidant stress to endothelial cells [34]. Interactions of modified haemoglobins with liposomal membranes and liposomes incorporating redox-active enzymes may provide insight into the nature of such interactions with physiological membranes of cells containing redox-active enzymes, which are attracted to those sites under oxidative stress, e.g. phagocytes, platelets and neutrophils.

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